Similar inductive effects of enamel and dentin matrix derivatives on osteoblast-like cell response over SLA titanium surface

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Abstract: OBJECTIVES The aim of this in vitro study was to investigate the behavior of osteoblasts on titanium discs under different concentrations of enamel matrix derivatives (EMD) and dentin matrix derivative (DMD). MATERIALS AND METHODS MC3T3-E1 osteoblast-like cells were cultivated on coated titanium SLA discs with EMD or DMD at 100 μg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml or left uncoated. Cell viability, proliferation, adhesion and migration were assessed respectively with MTT, BrdU, DAPI and scratch wound healing assays. Messenger ribonucleic acid of different genes related to osteoblastic differentiation was quantified by means of real-time quantitative PCR. Data were analyzed using student t-test for adhesion and migration assay and ANOVA for proliferation assay ($p < 0.05$). RESULTS BrdU incorporation was found in proliferative osteoblasts for both test solutions at all concentrations. Osteoblast migrated and covered approximately 70% of the wound area observed at time zero when exposed to EMD and DMD to all concentrations. The increase of gene expression was dependent on the concentration enhancement of EMD and DMD. Higher concentrations showed proliferation augmentation if compared to lower concentrations. CONCLUSIONS Roughness surface of Ti SLA can limit cell adhesion independent of the presence EMD or DMD. DMD enhances cell migration of osteoblasts on SLA titanium implants in a concentration-dependent manner.

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ABSTRACT

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Materials and methods: MC3T3-E1 osteoblast-like cells were cultivated on coated titanium SLA discs with EMD or DMD at 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml or left uncoated. Cell viability, proliferation, adhesion and migration were assessed respectively with MTT, BrdU, DAPI and scratch wound healing assays. Messenger ribonucleic acid of different genes related to osteoblastic differentiation was quantified by means of real-time quantitative PCR. Data were analyzed using student *t*-test for adhesion and migration assay and ANOVA for proliferation assay ($p < 0.05$).
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Conclusions: Roughness surface of Ti SLA can limit cell adhesion independent of the presence EMD or DMD. DMD enhances cell migration of osteoblasts on SLA titanium implants in a concentration-dependent manner.

1. Introduction

Dental implant success rate has been highly related to the direct functional connection or osseointegration between implant surface and bone (Bränemark & Zarb, 1988). Numerous experimental and clinical studies have shown that titanium (Ti) represents an implant material of choice due to its excellent biocompatibility, mechanical properties, and resistance to corrosion (Ratner, 2001; Thomsen, Larsson, Ericson, Sennerby, & Lausma, 1997). Many physical surface changes, such as, surface roughness modifications have been developed in order to achieve osseointegration. The surface character has been shown to play an important influential factor on the initial adhesion, osteoblast proliferation and subsequent extracellular matrix mineralization of the bone tissue. Besides changes on surface physical properties, proteins have been directly added to the surface of Ti as an attempt to chemically enhance ideal cell response. Among these proteins, the enamel dentin derivatives (EMD) has been extensively studied and used for periodontal regeneration (Hammarström, 1997). Several in vitro studies have demontrated the positive biological effects of EMD comparable to bone morphogenic protein-like activity. These effects were determined by enhancing proliferation, attachment and differentiation of many cell types, such as, periodontal cells, cementoblasts and osteoblasts (Bosshardt, Sculean, Windisch, Pjetursson, & Lang, 2005; Bosshardt, 2008; Johnson, Carnes, Steffensen, & Cochran, 2009). Moreover, animal experiments (Araujo & Lindhe, 1998; Sculean, Donos, Brecx, Reich, & Karring, 2000; Sculean, Donos, Reich, Karring, & Brecx, 1998) and clinical studies (Heden, Wennstrom, & Lindhe, 1999; Heijl, Heden, Svardstrom, & Ostgren, 1997; Rasperini & Ricci, 1999; Sculean, Reich, Chiantella, & Brecx, 1999) have demonstrated that EMD stimulates regeneration of periodontal tissue, including acellular cementum and alveolar bone (Bosshardt, 2008; Gestrelius, Andersson, Lidstrom, Hammarstrom, & Somerman, 1997; Hoang & Oates, 2000; Van der

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Pauw, Van den Bos, Everts, & Beertsen, 2000).

Extract of dentin matrix derivatives (DMD) have also been studied as natural source of bioactive proteins, which may control cell migration, proliferation and differentiation during physiologic developmental repair or regenerative processes of the dentin/pulp complex. More than 230 proteins have been identified within the DMD (Park et al., 2009; Smith, Duncan, Diogenes, Simon, & Cooper, 2016) and they include an array of growth factors like transforming growth factor β 1, non-collagenous proteins (phosphophoryn and sialoprotein), bone morphogenetic proteins and bioactive dentin phosphoprotein (Smith, Smith, Shelton, & Cooper, 2012, 2016). Recently, numerous in vitro and in vivo studies reported the tissue stimulating effects of soluble DMD on the promotion of proliferation and differentiation of dental pulp cells (Galler et al., 2016; Lee, Colombo, Ayre, Sloan, & Waddington, 2015). Enhancement of the mineral deposition of human stromal cell types from dental pulp was also noted after use of DMD (Petridis et al., 2018). In addition, DMD stimulates pulp regeneration in immature teeth with incomplete root formation (Galler et al., 2015). Thus, DMD seems of offer a valuable alternative source of endogenous growth factors, not only for endodontic regenerative therapeutic approaches, but also for periodontal bone regeneration. However, the understanding of the DMD role on periodontal regeneration and its influence on osteoblast-implant behavior is still largely lacking. Indeed, the initial interaction of osteoblasts with an implant surface include their attachment to the biomaterial surface (Brugge & Jansen, 2002). Moreover, their spreading could be greatly influenced by the use of DMD on the surface. Yet, for the most part, the exact role of the DMD biomarkers is still left to explore, since most previous research has been based on focusing on the in vivo DMD's biocompatibility, osteoconduction/osteoinduction capability and antibacterial properties (Smith et al., 2012). There is also a lack of knowledge on how DMD actually regulates cellular migration, which is a valuable question, as inadequate attachment and migration could result in inhibition of tissue regeneration (Brunette & Chehroudi, 1999).

Therefore, the present in vitro study aimed to examine the early interaction of osteoblast with a Ti implant surface after exposure to different concentrations of DMD in comparison with EMD. Specifically, morphology, attachment/adhesion and proliferation of osteoblasts was assessed after exposuse to DMD. Additionally, we have analyzed osteoblast differentiation and bone formation gene expression. We have shown that osteoblast cell behavior was comparable under influence of EMD and DMD, as both similarly enhanced osteoblast differentiation on Ti surface, in a concentration-dependent manner.

2. Material and methods

2.1. Titanium discs, enamel matrix derivatives and dentin matrix proteins

The study used sterile titanium (Ti6Al4V) discs of 10 mm diameter and 2 mm thickness provided by the Institute Straumann AG (Basel, Switzerland). The surfaces were sandblasted and acid-etched (SLA) according to a standard implant surface. The disc surface morphology analyzed by scanning electron microscopy (SEM, Zeiss Auriga, Carl Zeiss, Jena, Germany) and the surface topography was measured by using Talysurf Intra 50 profilometer (Taylor Hobson, Leicester, UK). The arithmetical mean peak-to-valley profile roughness parameters (Ra, Rz, Rt) were determined using a cut-off value of 0.8 mm and a measurement length of 4 mm. Discs underwent five measurements at different locations, and the average value of five discs was used as the measured roughness. In addition, the surface wettability was also analyzed with a contact angle meter (SL200, USA Kino Industry, Norcross, GA, USA) by using water drops for 3 s after application. Pictures of water contact angle were acquired (Image-Pro Plus version 6.0, Media Cybernetics Inc., Bethesda, MD, USA) and angles over 90° were considered hydrophobic. The average value resulting from these 6 discs was used as the measured wettability for each group. All discs were dipped in phosphate-buffered saline before distribution in 24-well plates before subsequent cell seeding.

EMD were prepared following the manufacturer's instructions (Institut Straumann AG, Basel, Switzerland). Various working concentrations of EMD (100 μg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml) were used during experimental seeding. EMD stocks were diluted in Dulbecco's modified Eagle Medium (DMEM, Invitrogen, Karlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Karlsbad, USA). Control samples were seeded without reagents containing medium with 10% FBS.

The dentin matrix proteins (DMD) were extracted from 10 canine porcine teeth. First, the pulp was removed with extirpation needles and K-files (Maillefer-Dentsply, Ballaigues, Switzerland) under sterile conditions. Then, the roots were separated from the crowns (Isomet low speed saw apparatus, Buehler LTD, Illinois, USA), and the roots were grinded for 4 min in a vibrating mill at 27 Hz (Retsch vibrating mill type MM 2000, Schieritz & Hauenstein AG, Arlesheim, Switzerland). The milling of the 10 canine porcine roots resulted in 9.78 g of ground root dentin. The particle size of the resulting powder was measured with the Cilas (Quantachrome GmbH, Odelzhausen, Germany) and was averaged 75 μm. The powder was then dissolved for 24 h in 50 ml of 0.5 M HCl and centrifuged at 300 g for 10 min (this process was repeated for four times). The final solution was then dissolved in 25 ml of 4 M guanidine – HCl solution (Fluka Chemie AG, Buchs, Switzerland) and kept at 4 °C for further 96 h under constant shake. After centrifugation for 5 min at 300 g, the supernatant guanidine-soluble dentine extract was dialyzed and lyophilized for 48 h (Freeze dryer alpha 2–4 LD plus, Millrock, NY, USA). The lyophilized yield of DMD was accounted for 11% of the total extract. DMD powder was added to the base medium DMEM containing 10% FBS to prepare solution concentrations of 100 μg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml.

2.2. Cell culture system

MC3T3-E1 osteoblast-like cells were donated from the Division of Cranio-Maxilo-Facial and Oral Surgery, Center of Dental Medicine, University of Zurich. Cells were seeded cell culture plates (5×10^4 cells/well) until the formation of 80% confluent cell monolayer using the DMEM supplemented with 10% FBS (Invitrogen, Karlsbad, USA), 100 units penicillin, and 100 μg/ml Streptomycin (Biochrom, Berlin, Germany) at 37 °C in a humidified atmosphere of 5% CO_2. For cell culture passages, the cells were washed with phosphate-buffered saline (PBS 1X) after achieved confluence and re-suspended with 0.25% trypsin (Seromond Biochrom, Berlin, Germany). Induction of differentiation to osteoblasts was promoted by culturing with 10 nM dexamethasone, 25 μm/ml L-ascorbic acid, and 10 mM glycerophosphate. The culture medium was replaced every 2 days and cells were used between passages 3 and 5.

2.3. Cell viability and proliferation

The influence of DMD on osteoblast viability was determined by the nonradioactive, colorimetric MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; Sigma, Steinheim, Germany). Tetrazolium bromide was fermented to formazan by viable cells and resulted formazan was measured after cell lysis photometrically. The osteoblasts were seeded over Ti discs (1×10^5 cells/well) on 24-well plates and incubated for 24 h at 37 °C. Then, the cells were separately treated for 24 h, 48 h and 72 h with EMD and DMD at concentrations of untreated (control), 100 μg/ml, 1 mg/ml, 10 mg/mL and 30 mg/ml. Then, 0.45 mg/mL of MTT was added to each well and the cells were incubated for 4 h at 37 °C. After incubation period, dimethyl sulfoxide was added on the cells as solubilization reagent and incubated for 2 h before absorbance reading at 570 nm with reference absorbance at 630 nm by using a spectrophotomer (Tecan, Austria, USA)

The cell proliferation rate was evaluated by measuring the 5-bromo-

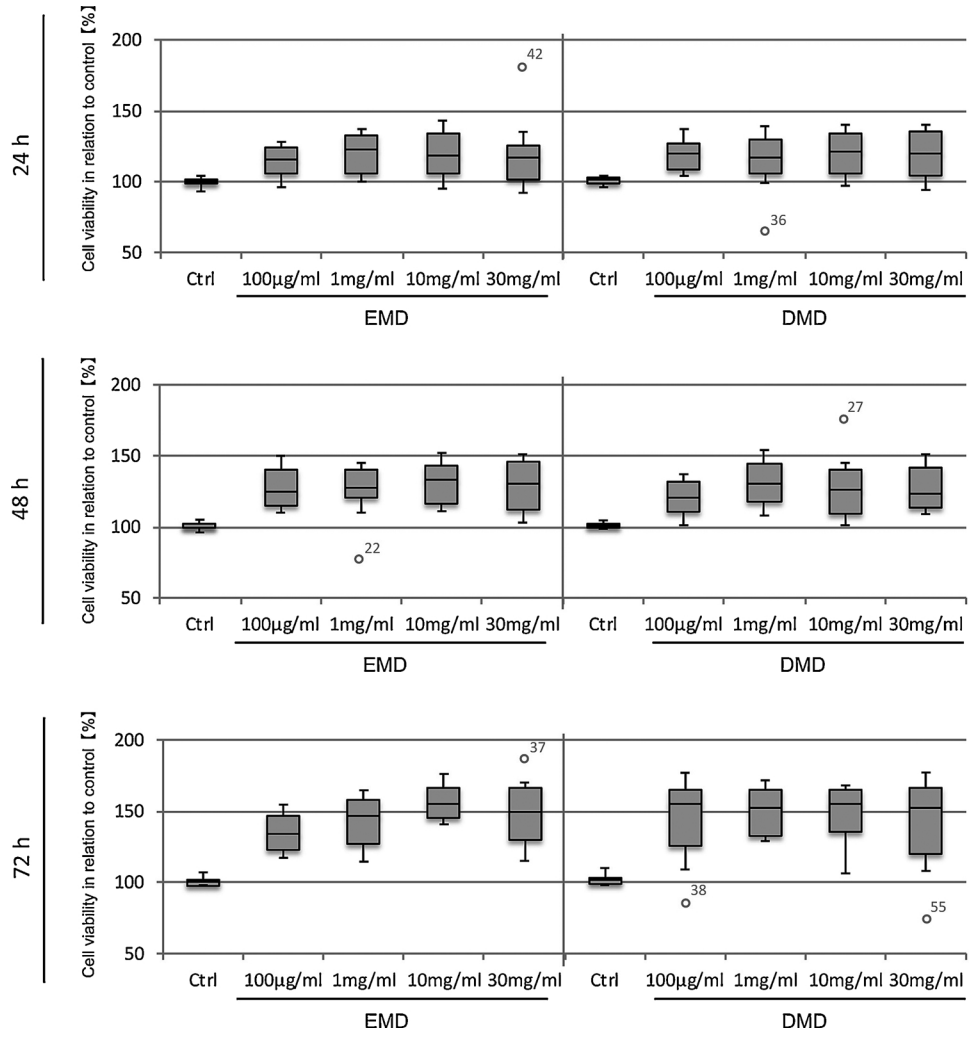


Fig. 1. Increase in cellular activity of osteoblasts 3 days after exposure. A significant increase in osteoblast cellular activity was detected for EMD and DMD concentrations of 110 µg/ml and higher treated cells. The ○ in the figure indicates the outliners. Data are shown of 3 samples (3 wells each) $p < 0.001$.

2′-deoxyuridine (BrdU) immunofluorescence incorporation into DNA during mitosis using a cell proliferation BrdU assay (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. BrdU incorporation was also assessed after 24 h, 48 h and 72 h of osteoblast culture on SLA Ti discs with untreated (control), 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml of EMD and DMD. Fluoroshield with DAPI (Sigma-Aldrich, Steinheim, Germany) was finally added and cover glasses were mounted on the Ti disc samples for image recording. Two samples of each group were used to perform the experiment and 3 images of each sample were taken with the confocal microscope (Leica DMI 4000B, Wetzlar, Germany). All experiments were performed in triplicate.

2.4. Cell adhesion, attachment and morphology

To assess the cell adhesion rate during the first 4 h after seeding, Ti SLA discs surfaces were coated with EMD or DMD (at same concentration of 100 µg/ml) and 1×10^5 cells/well were cultured in 24-well plates for 2 h, 4 h and 8 h. For each time point, the discs were washed with PBS and fixed for 10 min 4% formaldehyde. The cell number was measured by counting the stained nuclei with 4′, 6-diamidino-2-phenylindole (DAPI). A laser scanning confocal microscope was used for cell imaging (LSM710, Zeiss, Germany). The cells were counted at a magnification of 200x in five random fields on each sample. The attached cells were then removed from the surface by trypsinization and counted in the same way as a control. Uncoated discs were used as negative control. In order to quantify osteoblast attachment and morphology over Ti discs, disc surfaces were also analyzed by SEM (Zeiss Auriga, Carl Zeiss, Jena, Germany) after osteoblasts (1×10^5 cells/well in 24-well plates) treatment with EMD and DMD (at same concentration of 100 µg/ml) for 8 h. Before SEM protocol, cell culture plates and discs were washed in PBS 1X to remove non-adherent cells, then they were fixed with 4% glutaraldehyde fixative solution (Sigma, St. Louis, Missouri, USA) for 6 h. After removing the excess of fixative solution, PBS 1X washing was repeated twice. Then, cells were fixed with OsO_4 for 15–30 min. At the end, samples were dehydrated with series of ethanol bath (30%, 50%, 70%, 90% and 100%), dried and coated with a 100 nm thick layer of gold-palladium with an ion coater (Eiko IB-type 3). Images of the cells over SLA Ti surface were documented with SEM and recorded at 4k × and 10k × magnification. All experiments were performed in triplicate.

2.5. Cell migration

The in vitro migration or scratch wound healing model was used to compare the effect of DMD and EMD on the osteoblast cell migration without the use of SLA Ti discs. The cells were cultured (1×10^5 cells/well) in 24-well plate and incubated for 24 h at 37 °C until reached required confluence. Before scratch application and in order to inhibited mitosis, the cells were exposed for 2 h to 10 µg/ml of mitomycin

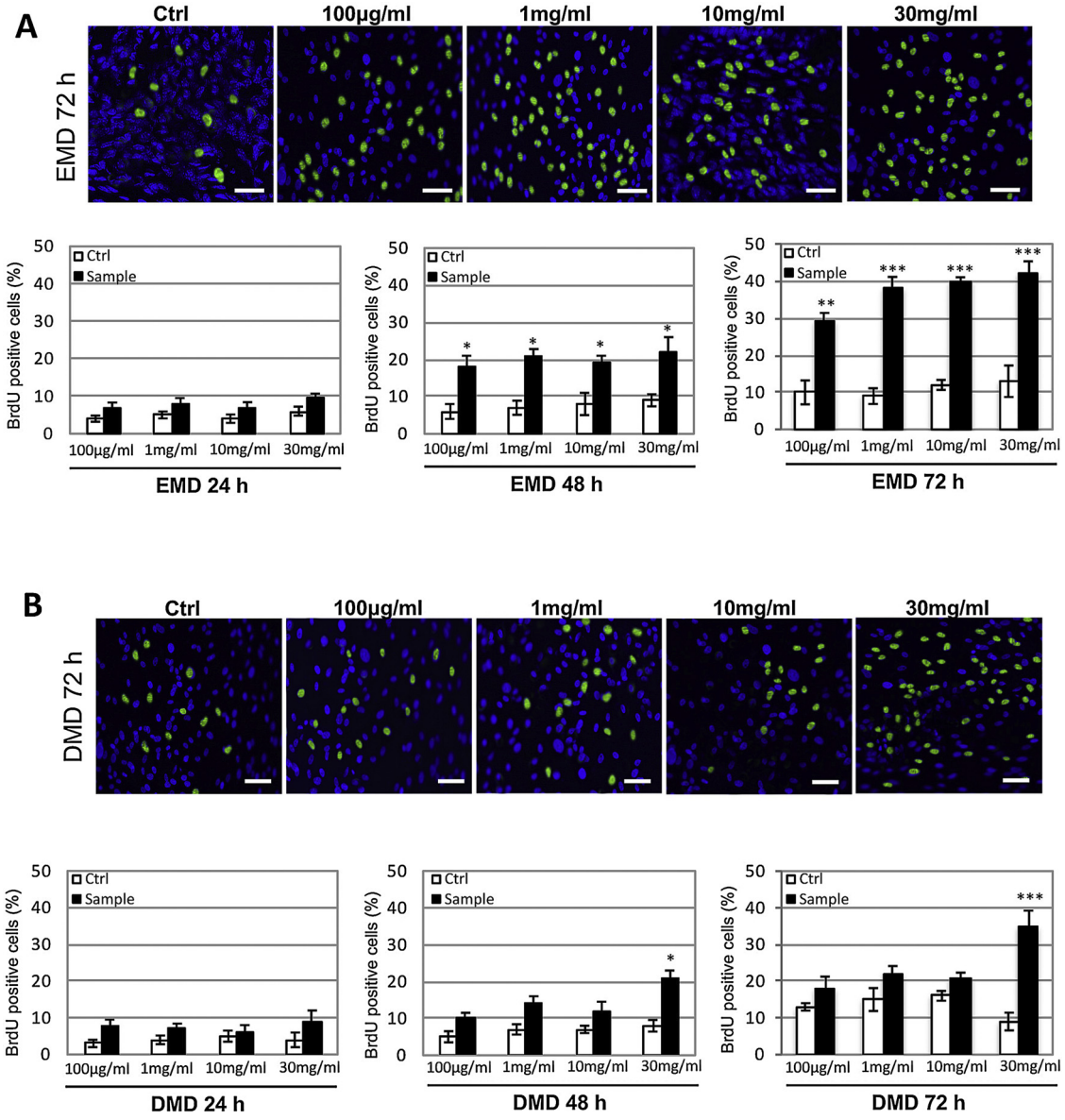


Fig. 2. Stimulation of osteoblast proliferation. Confocal laser scanning microscopic images of osteoblasts at 72 h. BrdU showed in fluorescent green and the nucleus in blue by diamidino-2-phenylindole (DAPI). Osteoblasts seeded over Ti disc samples showed significant increase in cell proliferation under concentrations of 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml of EMD. DMD treatment was able to increase proliferation only in 30 mg/ml. Representative images in the figure were taken with confocal Leica microscope. Experiments were performed in triplicate. Scale bar 50 µm (**$p < 0.05$, ***$p < 0.001$, mean ± SD) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

C (Sigma-Aldrich, Steinheim, Germany) in serum free medium. Using a yellow pipette tip of 10 µl, a scratch (700–900 µm in diameter) was created through the middle of the cell layer. Detached cells were removed by washing three times with phosphate-buffered saline and the remaining adherent cells were treated with non-supplemented DMEM (negative control), or with non-supplemented DMEM containing EMD and DMD at 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml. The cells were photographed every hour during 24 h, using an inverted light microscope equipped with a charge coupled device camera. The wound area was measured by evaluating photographed digital images of the wounded cell cultures (Olympus Camedia C-3000, Hamburg, Germany) and the area of the wound was determined using NIH Image Analysis System and Software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). The experiments were performed in triplicate and results were expressed as percentage of the original wound area.

2.6. Gene expression analysis for growth factors and differentiation markers

Gene expression for osteoblast differentiation and bone formation was investigated by Real-time quantitative PCR (RT-PCR). The osteoblasts were cultured over Ti discs (1×10^5 cells/well) on 24-well plates for 24 h with different concentrations of EMD and DMD (control, 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml). Then, the isolation of total RNA was conducted by using RNA Isolation Kit (Roche, Basel, Switzerland). The quantity of the total RNA (2 µg) was measured using a Biophotometer (Eppendorf, Hamburg, Germany) and converted into cDNA (RevertAid First Strand cDNA Synthesis Kit, Roche, Basel, Switzerland) according to manufacturer's protocol. The RT-PCR reactions were performed using Roche FastStart Universal SYBR Green Master and the 7500 real-time PCR System (Applied Biosystems, Grand Island, NY, USA) with the specific primers for genes encoding runt-related transcription factor 2 (Runx2) (forward primer: 5′-AGG GAC TAT GGC GTC AAA CA-3′, reverse primer: 5′-GGC TCA CGT CGC TCA TCT T-

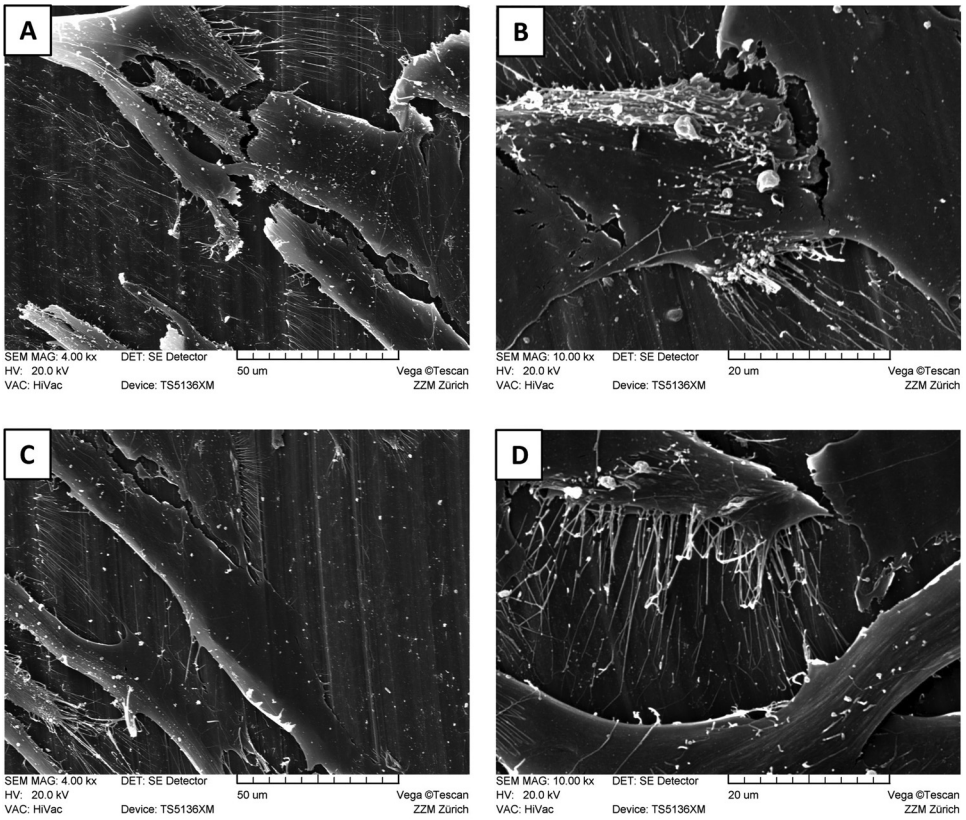
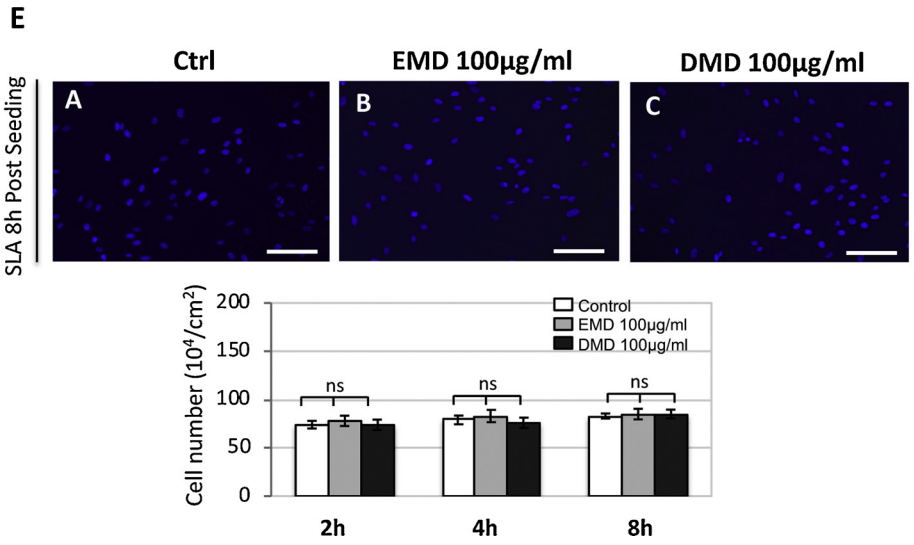


Fig. 3. No increase on osteoblast cell adhesion (DAPI), attachment and morphology (SEM microphotographs 8 h after seeding) over Ti SLA discs surfaces coated with EMD and DMD. A) Osteoblast on SLA Ti (4 k × magnification) treated with EMD, B) Osteoblast filopodia on SLA Ti (10 k × magnification) treated with EMD, C) Osteoblast on SLA Ti (4 k × magnification) treated with DMD, D) Osteoblast filopodia on SLA Ti (10 k × magnification) treated with DMD and E) Cell adhesion and density between the three experimental groups EMD and DMD (100 μg/ml) after 2 h, 4 h and 8 h of cell seeding (nucleai in blue by DAPI), scale bar 50 μm, mean ± SD (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).



3′); collagen1α2 (COL1A2) (forward primer: 5′- GGC TCC CAA CAC CGC TAA C-3′, reverse primer: 5′-GAT GTT CTG GGA GCC CTC AGT-3′); alkaline phosphatase (ALP), (forward primer: 5′-GGA CAG GAC ACA CAC ACA CA-3′, reverse primer: 5′-CAA ACA GGA GAG CCA CTT CA-3′); bone sialoprotein (BSP), (forward primer: 5′ – CCG GCC ACG CTA CTT TCT T-3′, reverse primer: 5′- TGG ACT GGA AAC CGT TTC AGA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer: 5′-AGG TCG GTG TGA ACG GAT TTG-3′, reverse primer: 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′). The relative mRNA expression of genes was normalized to the housekeeping gene GADPH and was analyzed using the comparative $2^{-\Delta\Delta CT}$ method.

2.7. Statistical methods

The mean values and standard deviations were computed for the MTT test and multiple comparisons were conducted by analysis of variance (ANOVA) with Bonferroni adjustment with a global significance level of 5% to assess the statistical significance of the differences between the experimental groups (SPSS version 22.0, Munich, Germany). All the in vitro experiments were performed in triplicates and from three independent experiments unless otherwise mentioned. Differences were considered significant at $p < 0.05$.

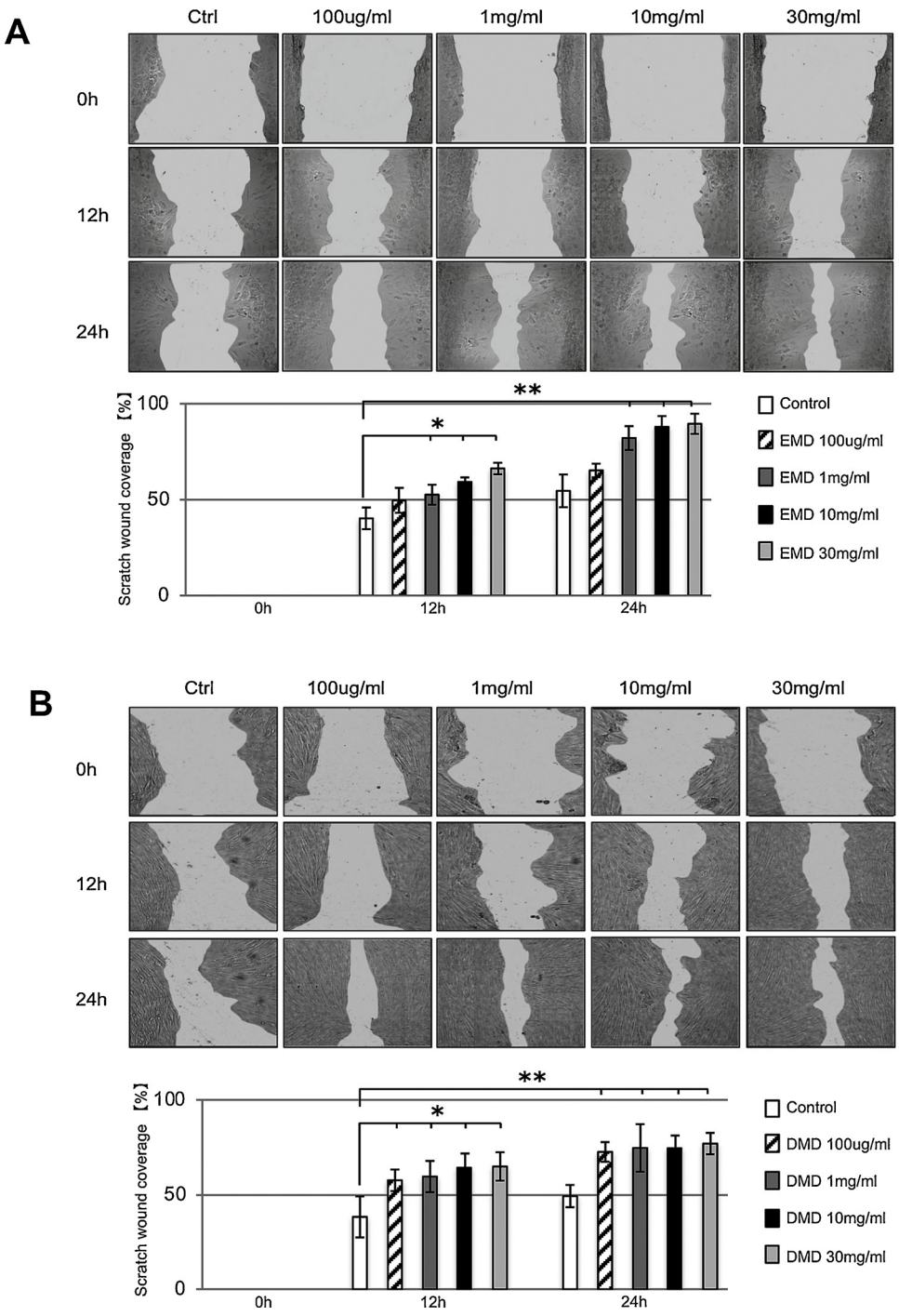


Fig. 4. Osteoblast cell migration in the presence of EMD and DMD. Wedges of the wound exhibit migration of cells into the wound area for EMD (A) and DMD (B). Migration of the cells from the original wound edges is expressed as the decrease in wound width, and migration distances during periods 0–12 h (migration during first 12-h period), 12–24 h (during second 12-h period) are shown separately. During hours 12–24 after wounding, a significant difference in migration distance was found for EMD at 1 mg/ml, 10 mg/ml and 30 mg/ml and for DMD in all concentrations. Data shown are representative figures from three independent experiments. (*$p < 0.05$, ** $p < 0.001$, mean ± SD).

3. Results

3.1. Surface topography analysis

Ti-discs presented the following roughness parameters: Ra (μm) ± SD (2.22 ± 0.276), Rz (μm) ± SD (4.87 ± 0.69) and Rt (μm) ± SD (6.24 ± 1.30). The wettability (median water contact angles) of Ti-discs accounted over 100°, which characterizes the hydrophobic Ti surface. The wettability testing did not change the surface roughness of the discs in any of the parameters measured.

3.2. Effect of EMD and DMD on osteoblast cell viability and proliferation

The MTT cell viability assay indicated no difference between test groups exposed to EMD and DMD at 24 h, 48 h and 72 h ($p = 0.0012$). Overall, the osteoblasts seeded onto the Ti SLA titanium surfaces showed similar degrees of viability (Fig. 1).

Similarly, osteoblast proliferation did not increase during the observation period of 24 h and 48 h after EMD and DMD treatment (Fig. 2A and B). At 72 h, BrdU incorporation was found in proliferative osteoblasts in all used concentrations of EMD (Fig. 2 A). In contrast, DMD treatment increased cell proliferation on SLA Ti discs only when cells were exposed to 30 mg/ml at 48 h and 72 h (Fig. 2B).

3.3. Effect of EMD and DMD on osteoblast cell adhesion, attachment and morphology

Images of SEM were used to record cell shape, growth and

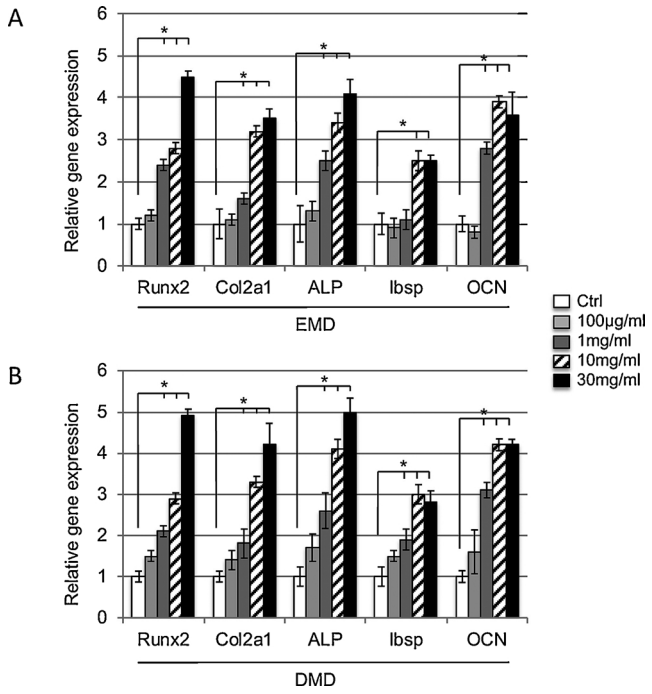


Fig. 5. EMD and DMD stimulated upregulation of osteoblast differentiation markers on osteoblasts. Quantitative PCR analysis shows expression increase of osteoblast *Runx2*, *COL1A2*, *ALP* and *BSP* under exposure to 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml of EMD (A) and DMD (B) compared to control (white bars). Upregulation was mostly found to be higher when exposed to 10 mg/ml and 30 mg/ml x-Axis: genes and concentration of EMD (A) and DMD (B); y-Axis: relative gene expressions. Data are mean ± S.D. of at least three independent experiments, *P < 0.05.

attachment of the osteoblasts seeded over SLA Ti surface (Fig. 3A-D) at 4k × and 10k × magnification. Cells treated with EMD and DMD (at same concentration of 100 µg/ml) presented parallel orientation to the direction of discs microgrooves after 8 h of seeding. No change in cell morphology or orientation was observed between EMD and DMD treated groups. Cell philopodia surface interactions was also found to be similar for both groups at same time period of 8 h (Fig. 3A-D). Great majority of cells were located inside the microgrooves with increased filopodia formation for both groups.

The evaluation of the cell adhesion by counting the DAPI stained nuclei revealed after 8 h of seeding no significant difference between the EMD and DMD group (Fig. 3E).

3.4. Effect of EMD and DMD on osteoblast cell migration

The osteoblast migration capacity the in vitro scratch wound-healing assay was determined in the presence of EMD or DMD and without SLA Ti discs. Images were acquired at 0 h, 12 h and 24 h after exposing the osteoblasts to EMD and DMD at 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml (Fig. 4A, B). After 24 h, osteoblasts migrated and covered approximately 70% of the wound area observed at time zero when exposed to DMD at concentrations of 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml. The wound closure in the EMD group with the concentrations of 10 mg/ml and 30 mg/ml mounted up to around 90% after 24 h. The negative control showed a closure of the wound area of about 50–55%.

3.5. Influence of EMD and DMD on gene expression of mineralization and ossification markers

Analyses on the expression levels of the *Runx2*, *COL1A2*, *ALP*, *BSP* genes in osteoblasts cultured for 72 h on SLA Ti discs previously coated with 100 µg/ml, 1 mg/ml, 10 mg/ml and 30 mg/ml of EMD (Fig. 5A) and DMD (Fig. 5 B) showed upregulation levels of expression of transcripts as compared to the negative control. Upregulation of genes was found to be higher when cells were exposed to the higher EMD and DMD concentrations of 10 mg/ml and 30 mg/ml.

4. Discussion

The purpose of this study was to ascertain the influence of different concentrations of DMD compared to EMD on osteoblasts seeded on Ti SLA. Taken together, our findings provide new evidence for the hypothesized benefit of DMD in combination with implant surfaces in regulating osteoblast behavior, especially at very initial stage. The results showed a similar increase osteoblast proliferation and differentiation on DMD-coated SLA surfaces compared to EMD-coated surfaces. A positive effect of DMD was also observered in previous study that compared EMD and DMD (specifically transforming growth factor β 1) on proliferation, mineralization and adhesion of periodontal ligament fibroblasts (Heng et al., 2015). In animal studies, DMD was also comparatively found to stimulate osteogenesis in rabbit calvarial defects combined with bone morphogenetic protein-2 (Um et al., 2016). In addition, autogenous DMD graft was generally found to be very effective as bone-forming materials for bone regeneration (Kabir et al., 2017). In particular regarding DMD properties of promoting osteoblast differentiation and osteoprogenitor cell proliferation in murine osteoblasts (Malaval, Liu, Roche, & Aubin, 1999). The results of cell migration response to EMD and DMD treatment without the use of SLA Ti discs show that, the effects at later time exposure (24 h) demonstrated that DMD was a more suitable for the use in lower concentrations (100 µg/ml) compared with EMD. These findings are intriguing because they suggest that the action of DMD components may be influenced by its concentration used on the cells, but also indicate that DMD contains bone-inducing factors that could maintain osteoinductive-healing capacities (Murata et al., 2010, 2011). Regarding osteoblast spreading on SLA Ti surfaces, previous studies have shown that osteoblasts cultured on SLA coated have a larger footprint and form more adhesions. In spite of that, overall surface roughness was still the determining factor dictating the level of cellspreading (Schuler, Trentin, Textor, & Tosatti, 2006). It was also shown that microgroove topography is more important than surface chemistry for guiding cell response (Britland et al., 1996). And it is likely that protein coatings may not have a large effect on cell spreading on rough topographies such as SLA. Although DMD has previously been reported to promote cell adhesion through an as yet unknown mechanism (Ravindran & George, 2015), it is unlikely to override the topographical features on SLA, as it is seem here in the present study. Furthermore, Runx2 (osteocalcin transcription factor) mRNA levels were increased. Therefore, it appears that both EMD and DMD accelerates the differentiation process by promoting a mature osteoblast phenotype. This was confirmed at the mRNA level as EMD and DMD pre-coated on SLA significantly increased ALP expression as well as mRNA levels of BSP and OCN. In fact, BSP has been hypothesized to act as a nucleus for the formation of the first apatite crystals (Harris et al., 2000).

This study has contributed to define the stimulating influence of DMD on osteoblasts proliferation confirming previous studies that demonstrated DMD as positive influencial factor on the bone metabolism. Additionally with the DMD extraction method used here, we may have in part proved the efficacy of DMD use as stimulating bone material extracted from teeth through easy handling, which may become a new promising product in dentistry. However, it is important to emphasize that methodological problems in the research design presented here may limit our interpretations. And the analysis of the experiments presented above emphasize the need for more nuanced framework of in vitro and in vivo studies for understanding the complete influence of DMD on dental implant success and long-term maintenance.

In summary, we have shown that EMD and DMD similarly enhance

osteoblast differentiation on Ti SLA surfaces in a concentration-dependent manner. DMD could be an important tool for enhancing bone formation around dental implants as proteins and growth factors from DMD may considerably improve bone response. EMD or DMD coating may represent a simplistic approach for increasing osteogenesis around metals such as Ti. Further investigations on this topic are now open for scrutiny.

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